

Developing Meaningful Assays for the Eukaryotic Transcription Reagents TFIIB and Anti-TFIIB

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The general transcription factor TFIIB is an integral component of the RNA polymerase II initiation complex. Promega now offers human recombinant TFIIB and an Anti-TFIIB monoclonal antibody. In this article, we describe the functional assays we developed for this factor and antibody. We also compare our preparations of recombinant TFIIB with those from another supplier and demonstrate the higher degree of purity and activity of the Promega preparations.

Introduction

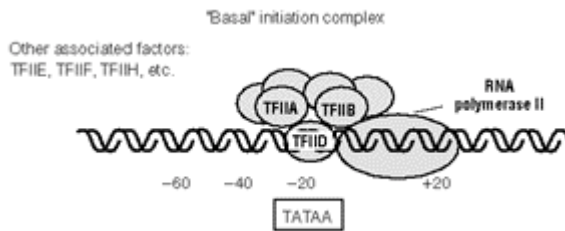
Advancements in the understanding of transcriptional control mechanisms have been facilitated by the use of reagents such as transcriptionally active nuclear extracts, recombinant transcription factors and antibodies. These reagents may be used to assess DNA-protein interactions, protein-protein interactions and the effects of expression during transient or stable cell transfections. Unfortunately, the activities of reagents can differ significantly between laboratories, making results difficult to reproduce.

Thus, standardized, widely-available reagents such as transcription factors and antibodies are necessary for accelerating the progress of eukaryotic transcription research. The design of meaningful assays for basal transcription factors is complicated by the multiple functions that these regulatory molecules exhibit. A further complication is that transcription factors expressed as recombinant proteins in *E. coli* often contain a substantial proportion of inactive protein. Realistically, a functional assay must strike a balance between simplicity of design and accurate measurement of the factor's activity in a relevant biological context. Promega is dedicated to developing meaningful, functional assays for transcription factors and related reagents for eukaryotic transcription research. This article describes the development of assays for two new reagents now available in Promega's Eukaryotic Transcription Regulation product line: human recombinant TFIIB and an Anti-TFIIB monoclonal antibody.

Role of TFIIB in basal transcription initiation

TFIIB is a general transcription initiation factor, approximately 35kDa, which performs a central role in RNA polymerase II transcription of genes (1). The current paradigm for the assembly of the RNA polymerase II initiation complex envisions the binding of TATA-Binding Protein (TBP) of the TFIID complex at a TATA box, possibly with the cooperation of TFIIA, followed by the binding of TFIIB to form a D-B (or D-A-B) complex (Figure 1). RNA polymerase II, along with TFIIF, then associates with the D-B (or D-A-B) complex, followed by other factors such as TFIIE and TFIIF (2,3). In functional activity assays, reconstitution of basal transcription *in vitro* on a supercoiled immunoglobulin promoter required only the purified factors TFIIB, TBP and RNA polymerase II (4). The general factor TFIIB also associates with transcription factors other than those in the RNA polymerase II initiation complex,

suggesting that it plays a broader role in mediating activation. For example, direct protein-protein interactions of TFIIB have been demonstrated with the acidic activation region of VP16 (5), members of the steroid hormone receptor family (6), and the *Drosophila fushi tarazu* activation domain (7).



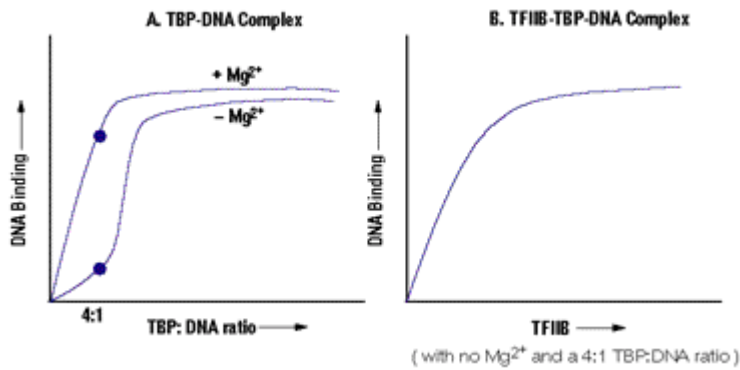
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Figure 1. Paradigm for an RNA polymerase II transcription initiation complex.

Assay for TFIIB: Modeling an early step of basal transcription complex formation

Given the multifunctional activities of TFIIB, it was a challenge to devise a meaningful unit activity definition for this protein. We needed a simple assay with defined components that would accurately measure its role in basal transcription complex formation. Unlike the other recombinant transcription factors that Promega provides (TBP, SP1, c-Jun, AP2 and the NF-kappa-B p50 and p49 subunits), TFIIB does not bind to a DNA template. Therefore, direct measurement of DNA binding activity by footprint or gel mobility shift analysis was not possible. Instead, we took advantage of the observation that the relatively unstable association of TBP with a TATA-containing oligonucleotide in gel mobility shift assays (8) can be stabilized in the presence of TFIIB. Using this information, we created a "gel shift unit" definition for TFIIB based upon only three components: TFIIB, a TATA box consensus oligonucleotide and TBP.

The first stage of this assay establishes the binding activities of TBP required to fully shift 0.2pmol of the TATA-containing oligonucleotide in 25µl of binding buffer containing MgCl₂. Next, binding reactions are performed in the absence of MgCl₂. In the absence of Mg ions, the binding of TBP to DNA is weakened enough that not all of the DNA shifts, even though the previous experiment established that a stoichiometric amount of active TBP is present. In the example shown in [Figure 2](#), panel A, the Mg-dependence of binding is most evident at a 4:1 ratio of TBP:DNA. When TFIIB is added to such a reaction mixture lacking Mg ions, binding activity is restored ([figure 2](#), panel B). By titrating the added TFIIB, an activity unit can be determined. Although this unit is designated as a gel shift unit, in this case it really is a TBP-binding unit as detected by gel shift. One gel shift unit of TFIIB is defined as the minimal amount of TFIIB which maximizes the level of TFIIB-TBP-DNA complex defined reaction and gel mobility shift assay conditions.



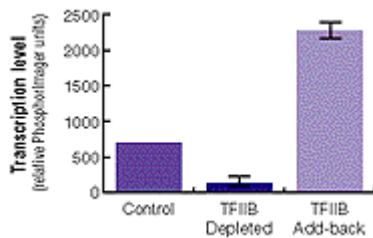
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Figure 2. Example of a TFIIB complex formation assay.

Since the TBP-DNA complex and the TFIIB-TBP-DNA complex have similar gel mobilities and dissociate somewhat during the electrophoresis run, the binding activity of TFIIB is actually measured by the decreasing levels of unshifted, or "free," oligonucleotide rather than the increasing levels of the shifted TFIIB-TBP-DNA complex. The presence of TFIIB and TBP in this complex has been verified by "supershifting" to an apparently higher molecular weight complex using Anti-TFIIB or Anti-TBP (data not shown).

Transcriptional activity assay for TFIIB

The ability of recombinant TFIIB protein to activate transcription is also important to the functionality of the protein. Promega tests the ability of the recombinant TFIIB to restore transcriptional activity in nuclear extracts depleted of TFIIB activity (Figure 3). To prepare the depleted extract, HeLaScribe(TM) Nuclear Extract was incubated with Anti-TFIIB coupled to *Staphylococcus aureus* cells, and the TFIIB-antibody complex was precipitated. Transcriptional activity from the HIV LTR promoter linked to a G-less cassette (10) was evaluated under standard conditions using the HeLaScribe Extract, TFIIB-depleted extract, and TFIIB-depleted extract supplemented with 50ng of recombinant TFIIB. As shown in Figure 3, the addition of TFIIB increased the level of transcription more than 15-fold above the activity measured in depleted extract alone.



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Figure 3. TFIIB transcriptional activation in HeLaScribe Nuclear Extract. *In vitro* transcription reactions were performed as described in reference 9. Each reaction contained 0.3 μ g of plasmid DNA containing the HIV LTR promoter, [α - 32 P]UTP, 3' O-methyl-GTP and HeLaScribe Nuclear Extract, and was incubated at 30°C for 90 minutes. After treatment with Rnase T1, the transcription products were resolved on a 6% polyacrylamide gel and quantitated using a Molecular Dynamics PhosphorImager(TM). Bars indicate the high and low values of replicate samples.

Vendor comparison of TFIIB purity and activity

In addition to the activity assays, a standard protein analysis is performed on Promega's recombinant TFIIB. The total protein concentration for each lot is documented, along with its relative purity as observed in a Coomassie®-stained SDS-polyacrylamide gel. [Figure 4](#) illustrates the purity of Promega's TFIIB relative to a preparation of TFIIB obtained from another vendor. It is apparent from [Figure 4](#) and [Figure 6](#) that there is a major contaminating band at approximately 14kDa, which reacts with Vendor B's polyclonal antibody against TFIIB. This contaminant may be a proteolytic fragment of the authentic 35kDa TFIIB (11). The presence of a TFIIB fragment could generate artifactual results in binding or transcription assays.

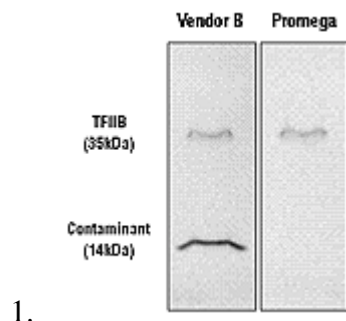


Figure 4. SDS-Page analysis of TFIIB purity. Samples were resolved on a 4-20% SDS-polyacrylamide gel and stained with Coomassie blue. The gel lanes were loaded so as to contain approximately equivalent amounts (200ng each) of the 35kDa TFIIB band.

The functional activity of TFIIB from Promega and from Vendor B was compared using the gel shift assay described above. The maximal amount of shifted oligonucleotide was obtained using 0.7pmol of Promega TFIIB ([Figure 5](#)). Only 33% of this activity was observed when an equivalent amount of Vendor B's TFIIB was used in this assay.

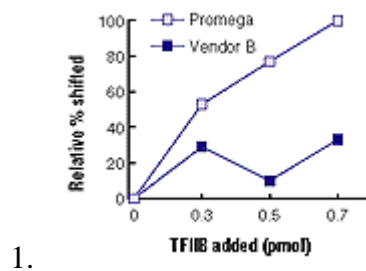


Figure 5. Vendor comparison of TFIIB gel shift activity. Gel shift assays were performed as described in this article. Equimolar amounts of TFIIB from Promega or Vendor B were added, using the amount of protein detected in the 35kDa band as the basis for comparison.

Promega also routinely tests transcription factor preparations for DNase and RNase activities. Any DNase activity in the transcription factor preparations could certainly confound results obtained from footprinting and gel mobility shift assays, and Rnase contamination could adversely affect studies in which the factor is added to reconstituted systems for transcriptional assays.

Anti-TFIIB monoclonal antibody

Specific antibodies against general transcription factors can be used for several purposes. For example, an antibody can be used to follow the purification of a factor from nuclear extracts or recombinant sources, to purify with antibody-coupled resins, to immunoprecipitate and to verify the presence of the factor in gel shifted complexes. Promega's monoclonal antibody to TFIIB is suitable for many applications, including those listed in [Table 1](#).

Western blot assays using Promega's and Vendor B's Anti-TFIIB are shown in [Figure 6](#). Vendor B's polyclonal antibody reacts strongly with recombinant TFIIB and with the contaminating 14kDa band in Vendor B's TFIIB preparation (lane 1), supporting the suggestion that this band is related to full-length TFIIB. In contrast, Promega's monoclonal Anti-TFIIB recognizes only the 35kDa TFIIB band in both preparations.

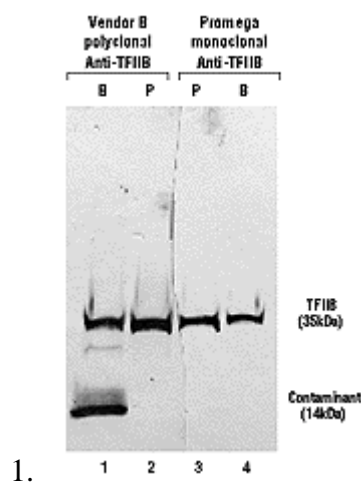


Figure 6. Specificity of Anti-TFIIB antibodies. Samples were resolved on a 4-20% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Sample loadings were 1µl of either Vendor B's (B) or Promega's (P) human recombinant TFIIB, approximately 50ng of the 35kDa band (lanes 1-4). Immunodetection was performed by incubation with a 1:500 dilution of either Anti-TFIIB Polyclonal IgG (Vendor B) or Anti-TFIIB Monoclonal IgG (Promega), followed by a 1:2,000 dilution of either Rabbit Anti-IgG or Mouse Anti-IgG Alkaline Phosphatase Conjugate, respectively. Bound antibody complexes were visualized using Western Blue(TM) NBT/BCIP Substrate.

Table 1. Properties of Anti-TFIIB Antibody

Form	Mouse monoclonal IgG2a
Suitable applications	Western and dot blots, immunoprecipitation, immunopurification with soft-release properties (12), "supershifting" TFIIB-TBP-DNA complexes.
Reactivity	Reacts predominantly with one 35kDa band in HeLaScribe Nuclear Extract. Crossreacts with human and mouse TFIIB.
Nuclease contamination	RNase and DNase below levels that would affect gel shift or transcription assay.

Summary

Many parameters must be considered when designing assays to evaluate the functionality and consistency of transcription factors and their respective antibodies. Our goal is to set the standard of quality for transcription factors in transcription regulation research. As new applications for transcription factors evolve in the future, we will continue to respond by developing the relevant functional assays needed to support this rapidly developing field.

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Ordering Information

Product	Size	Cat.#
TFIIIB (human)	50gsu	E3790
Anti-Human TFIIIB, IgG, (mono)	50µg	E3830
TFIID (human), TATA-Binding Protein	50fpu	E3081
Anti-Human TBP, IgG, (mono)	50µg	E4151

gsu = gel shift unit

fpu = footprint unit

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